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Mutations in v-Src SH3 and Catalytic Domains That Jointly Confer Temperature-Sensitive Transformation with Minimal Temperature-Dependent Changes in Cellular Tyrosine Phosphorylation

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We have analyzed two functionally significant amino acid alterations encoded by the temperature-sensitive (*ts*) v-src mutant of Rous sarcoma virus, LA32. The G-to-V change at residue 300 in the catalytic domain nonconditionally impairs morphological transformation, in vitro kinase activity, in vivo tyrosine phosphorylation, and the cytoskeletal association of v-Src while rendering anchorage- and serum-independent growth *ts*. The R-to-P mutation in the SH3 domain subtly enhances morphological transformation but has no phenotype if the catalytic domain is inactivated. In the presence of the G-300-to-V mutation, this SH3 domain lesion does not affect v-Src in vitro kinase activity and cytoskeletal association, but it nonconditionally enhances cellular tyrosine phosphorylation and restores morphological transformation at the permissive temperature only. This ability to induce a *ts* transformed morphology, in concert with nonconditional elevations of cellular phosphotyrosine, suggest that a subset of v-Src targets that are crucial to transformation may be affected in *ts* fashion by the SH3 mutation. Consistent with this, we find that the R-107-to-P mutation confers *ts* activity and tyrosine phosphorylation on the SH3-binding enzyme phosphatidylinositol 3'-kinase. Thus, both the SH3 and catalytic domain mutations in LA32 have some *ts* attributes and they cooperate in determining the mutant's behavior. The *ts* SH3 mutation is unique and offers the potential for deeper understanding of the function of this domain.

The v-src oncogene product, pp60^{v-src} (v-Src), is a non-receptor protein tyrosine kinase that comprises several domains, including a catalytic region (amino acids 260 to 514) and Src homology (SH) domains, SH2 (amino acids 140 to 250) and SH3 (amino acids 85 to 139). There is abundant evidence that v-Src exerts all or most of its neoplastic effects on cells through its kinase domain activity, but it is also apparent that the consequences of this activity are modulated, and presumably given specificity, by other domains, including SH2 and SH3. The SH2 and SH3 domains (reviewed in references 16, 21, and 22) bind phosphotyrosine-containing peptides and proline-rich peptides, respectively, and are found in many proteins, notably the small adaptor proteins such as Crk. Their domain structures have recently been determined (36, 41). These findings encourage the view that both domains are self-contained modules that determine the interactions of proteins that contain them but that do not affect the inherent structures or functions of contiguous protein domains. However, both SH2 and SH3 domains appear to participate in intramolecular interactions that maintain c-Src kinase in an inactive state (20, 30). There are thus unsubstantiated possibilities in v-Src for these domains to interact with one another or with the catalytic or other regions of the protein (1, 34).

This conjecture rekindled our interest in a temperature-sensitive (*ts*) v-src mutant of Rous sarcoma virus (RSV), LA32, which encodes alterations in both the SH3 domain (R at amino acid 107 to P [R107P]) and the kinase domain (G at position 300 to V [G300V] and R at 419 to Q [R419Q]) (40). In chicken

embryo fibroblasts (CEF) at the permissive temperature (35°C), this mutant protein induced anchorage-independent growth in agar, and the morphology of transformed cells on a substrate was refractile, but fusiform rather than round. At the restrictive temperature (41°C), agar growth was much reduced and the cellular morphology resembled that of uninfected CEF (28). However, although both the in vitro tyrosine kinase activity and the plasma membrane association of this protein were reduced, neither was markedly *ts*, nor was there any discernible defect in the biogenesis or amino-terminal myristylation of LA32 v-Src at either temperature (29). Moreover, the candidate v-Src substrates calpactin and vinculin were phosphorylated at both 35 and 41°C, and there was no temperature sensitivity in total cellular phosphotyrosine levels (14, 28).

These initial findings indicated that neither the catalytic activity nor the gross localization of LA32 v-Src differed markedly between permissive and restrictive conditions. We therefore postulated that the *ts* phenotype resulted, at least in part, from subtle changes in the protein's cellular interactions, possibly mediated by the SH3 domain mutation. This report examines that hypothesis by analyzing the individual and combined effects of the SH3 and kinase domain mutations on the biochemical attributes and biological effects of v-Src. Many analyses were performed in Rat-1 cells as well as CEF, and since where this was done the results were similar in both hosts, we present examples of data from both species. These data showed that the G300V mutation constitutively impaired the function of the kinase domain and consequent cellular transformation but transformation was restored by the R107P lesion, and under these conditions, both mutations could contribute to the temperature sensitivity of the phenotype. In addition, although cellular tyrosine phosphorylation was elevated at the restrictive temperature, at least one candidate target of v-Src was shown to be *ts* in function and tyrosine

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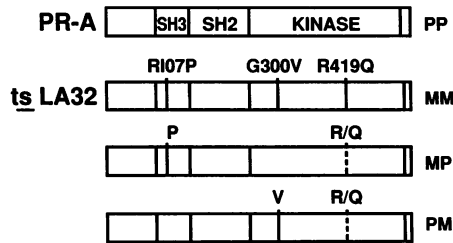


FIG. 1. Diagrammatic domain structure of RSV wild-type (PP) Prague A (PR-A) strain v-Src and the position of mutations in *ts* mutant LA32 (MM) and chimeric MP and PM proteins. R/Q indicates that the presence of either arginine or glutamine at this site is immaterial to the protein's behavior.

phosphorylation in a manner dependent on the SH3 domain mutation.

MATERIALS AND METHODS

Cells, plasmids, and viruses. CEF were prepared from 10-day-old White Leghorn embryos (Wickham Laboratories, Hampshire, England). Culture, including focus and soft agar growth assays, employed standard techniques of this laboratory (31, 39).

The *v-src* genes of the wild-type Prague A strain of RSV and its *ts* mutants LA29 and LA32 had previously been cloned in this laboratory (7, 27, 37). For introduction of these genes into mass cultures of CEF, full-length viruses were constructed by using a pRAV vector (33) as previously described (37), and 10^6 cells were transfected with 1 μ g of plasmid DNA by using transfection reagent (Boehringer Mannheim, Lewes, England). To obtain Rat-1 cell lines, the *v-src* genes were cloned into the selectable mammalian vector fpGV-1 (24) (kindly provided by G. S. Martin), and 10^6 cells were transfected with 5 μ g of plasmid DNA and then subjected to selection with G418 (Gibco, Paisley, Scotland) and cloning by micromanipulation.

Chimeras between wild-type and LA32 *v-src*, as depicted in Fig. 1, were made by subcloning appropriate combinations cut at the *v-src* *Bgl*II site (nucleotide 7736). Mutant *v-src* with an inactive kinase was constructed by replacing the K295 codon with an R codon, using PCR techniques (32). The identity and integrity of all clones were confirmed by sequencing.

Cell fractionation. The separation of detergent-soluble and cytoskeletal cell fractions was done as described previously (37). Briefly, cells at the appropriate temperature were labelled for 3 h with [35 S]methionine (Amersham International, Amersham, England) at 100 μ Ci/ml and then incubated thrice with MCSK buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 6.8], 3 mM $MgCl_2$, 50 mM NaCl, 0.3 M sucrose, 0.5% Nonidet P-40 [NP-40], 1 mM $CaCl_2$) on ice to yield the detergent-soluble fraction. The residual fraction was solubilized in NP-40 buffer (1% NP-40, 150 mM NaCl, 20 mM Tris-HCl [pH 6.8], 20 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 1 μ g of aprotinin per ml). Src was immunoprecipitated with monoclonal antibody JB327 (Oncogene Science, Cambridge, England), resolved on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels, and visualized by impregnation with Amplify (Amersham) followed by drying and autoradiography.

In vitro kinase assays. Cells were lysed in NP-40 buffer and clarified, and v-Src was immunoprecipitated with antibody

JB327. The washed precipitate was incubated for 5 min at 30°C with 5 μ Ci of [γ - 32 P]ATP (3,000 Ci/mmol; Amersham) and 8 μ g of rabbit muscle enolase (Sigma, Poole, England) in kinase buffer [100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 2 mM $MnCl_2$, 10 μ M sodium orthovanadate]. The reaction was stopped with NP-40 wash buffer (10 mM Tris-HCl [pH 8], 400 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate), the precipitates were washed thrice, and substrates were resolved on SDS-10% polyacrylamide gels.

Phosphoamino acid analysis. The analysis was done by the method of Cooper et al. (3). Briefly, cells in phosphate-free medium, supplemented with 5% dialyzed newborn calf serum, were incubated for 18 to 20 h in carrier-free $^{32}P_i$ (1 mCi/ml; Amersham), washed, and lysed on ice in modified radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 7], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β -glycerophosphate, 10 μ M sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 1 μ g of aprotinin per ml). After clarification, proteins were extracted and hydrolyzed, and hydrolysates, together with unlabelled phosphoamino acid standards, were spotted on a cellulose thin-layer plate (20 by 20 cm; Kodak). Following electrophoresis in two dimensions at pH 1.9 and 3.5, respectively, standards were visualized with ninhydrin to identify areas for scraping and counting.

Protein blotting. Cells were lysed in modified RIPA buffer (as described above but with phosphate-buffered saline in place of Tris-HCl), and protein concentrations were determined by the Micro BCA method (Pierce, Chester, England). For direct analysis of v-Src and phosphotyrosine, 25 or 50 μ g of protein was resolved on SDS-10% polyacrylamide gels, transferred to nitrocellulose, and probed with either v-Src monoclonal antibody EC10 (UBI, TCS Biologicals, Botolph Claydon, England) or antiphosphotyrosine monoclonal antibody Py20 (UBI). To examine phosphotyrosine levels in the p85 subunit of phosphatidylinositol (PI) 3'-kinase, 500 μ g of RIPA lysates was first immunoprecipitated with polyclonal p85 antibody (UBI). Detection was by enhanced chemiluminescence (Amersham) as instructed by the manufacturer.

PI 3'-kinase assay. Cells were grown to 90% confluence on rat tail collagen 1 (Strattech, Luton, England) at the appropriate temperature, washed, and rapidly lysed in PLC lysis buffer (15). Clarified lysates were incubated overnight with protein A-Sepharose beads (Sigma) coated with v-Src monoclonal antibody EC10 (UBI), control normal mouse immunoglobulin G (ICN, High Wycombe, England), or polyclonal p85 antibody (UBI). Washed immunoprecipitates were then incubated in assay buffer (50 mM HEPES, 10 mM NaCl, 1 mM EDTA [pH 7.4]) with a 3:1 mixture of PI and phosphatidylserine (0.02 mg/ml) and with 50 μ M ATP, 50 μ Ci of [γ - 32 P]ATP, and 10 mM $MgCl_2$ as substrates for 30 min at 30°C. Reaction products were extracted and separated by thin-layer chromatography. After autoradiography, the PI 3-phosphate band was scraped off the plate and counted.

RESULTS

Mutations in v-src. The three mutations of LA32 were examined singly and in all combinations of doubles in a variety of biological and biochemical tests. The mutant proteins were designated by a two-letter code which identified a mutant (M) or wild-type (P) amino acid at position 107 (first letter) or 300 (second letter) (Fig. 1). The mutation at position 419 has no apparent biological significance, since in all tests, the amino

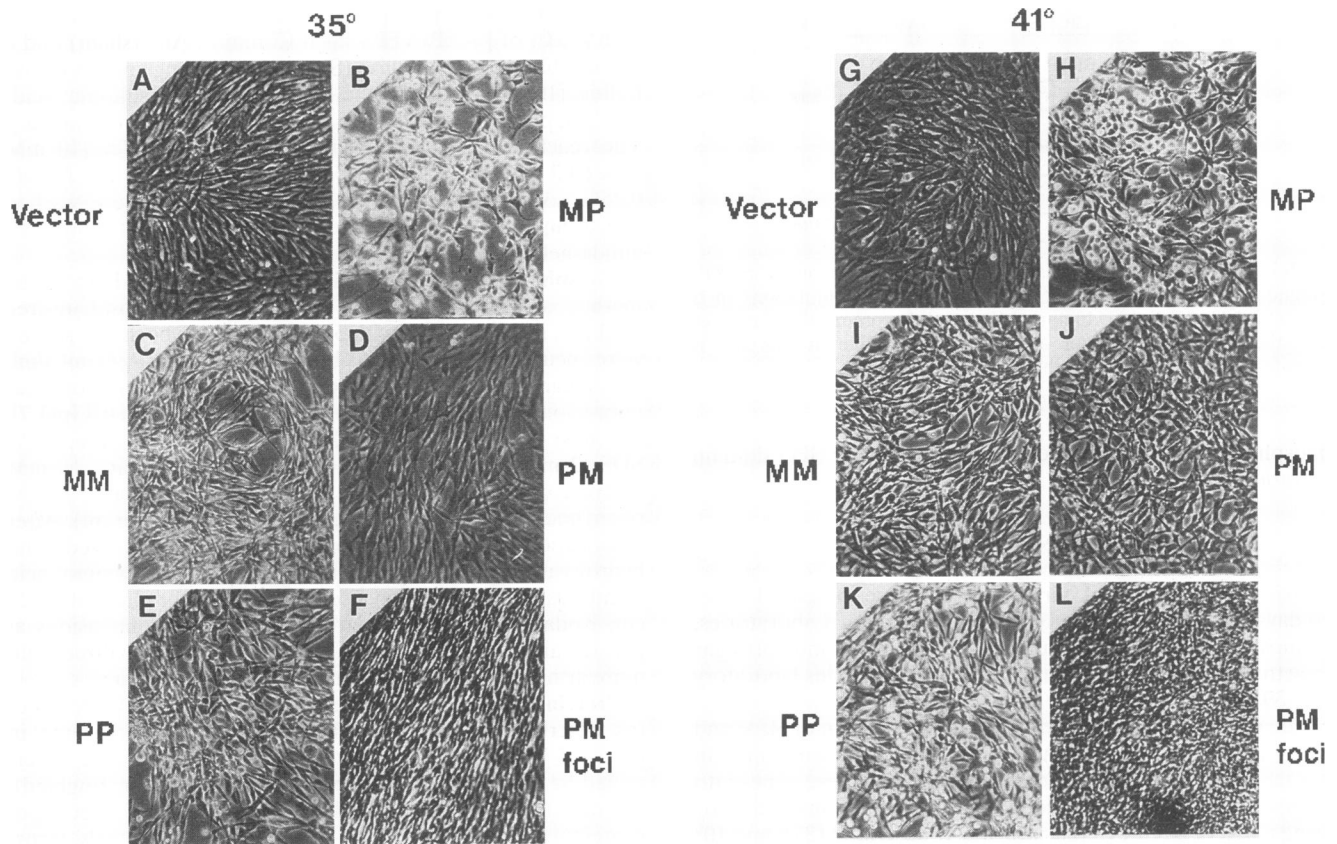


FIG. 2. Morphologies at permissive (35°C) and restrictive (41°C) temperatures of mass cultures of CEF after transfection with the pRAV vector or constructs bearing chimeric v-Src proteins as identified in Fig. 1. Cultures in panels F and L were infected for several days longer and maintained under an agar overlay.

acid at this position was immaterial to the result, and its identity is not included in the mutant designation.

Biological effects of v-src mutations. As previously shown (28), MM v-Src proteins induced a morphology that was transformed but fusiform at 35°C and similar to that of uninfected chick cells at 41°C (Fig. 2A, C, G, and I). PP proteins induced transformation at both temperatures (Fig. 2E and K) and chimeric MP proteins were also markedly transforming at both 35 and 41°C (Fig. 2B and H). Indeed, the latter induced a more rounded cellular phenotype in cultures, which could be maintained in an attached state only in collagen-coated dishes. In contrast, PM v-Src induced very little morphological change at either temperature (compare Fig. 2D and J with Fig. 2A and G, respectively). However, when cultured under focus assay conditions (a week or more under agar), PM chimeras elicited macroscopically visible thickenings of the cell layer at 35 but not 41°C. These foci contained aligned and somewhat refractile cells, contrasting with the indistinct cell boundaries in cultures kept at 41°C (Fig. 2F and L).

Anchorage-independent growth in agar partly paralleled the morphological changes, with PP and MP v-Src inducing large colonies at both temperatures, although those induced by MP were more compact at the restrictive temperature (Fig. 3C to F). MM chimeras were *ts* for growth (Fig. 3A and B), while PM proteins, although inducing only slight morphological changes, were clearly also capable of inducing growth in agar which was *ts*, but less obviously so than that induced by MM (Fig. 3G and H). In common with other *ts* transformation mutants of v-Src,

cells containing MM and PM v-Src were arrested in the G₀ phase of the cell cycle by serum deprivation at the restrictive temperature (reference 38 and references therein). When shifted to permissive conditions, the MM chimera induced cell cycle progression with kinetics similar to those produced by addition of 5% serum. Cells containing the PM chimera, but not control cells containing vector alone, also progressed significantly through the cell cycle at 35°C, although the effect was less than for MM (data not shown).

These results show that the LA32 G300V mutation impairs morphological transformation at both permissive and restrictive temperatures and renders the virus partially *ts* for growth-promoting activity, as judged by focus formation and anchorage- and serum-independent growth. The R107P mutation has a compensatory effect, restoring morphological transformation at the permissive temperature but not affecting, and indeed possibly augmenting, the temperature sensitivity mediated by the mutation at position 300.

Localization of v-Src. When the subcellular distribution of LA32 (i.e., MM) v-Src protein was determined by fractionation of Dounce-homogenized CEF, the proportion in the crude membrane pellet was reduced by about 5% at 35°C and by about 40% at 41°C compared with the wild-type (PP) protein (29). The partitioning of chimeric proteins between the soluble and cytoskeletal cellular compartments was therefore analyzed. We found that the reduced cytoskeletal association of MM proteins was shared by PM chimeras, while both MP and PP proteins were mainly cytoskeletal under the conditions of

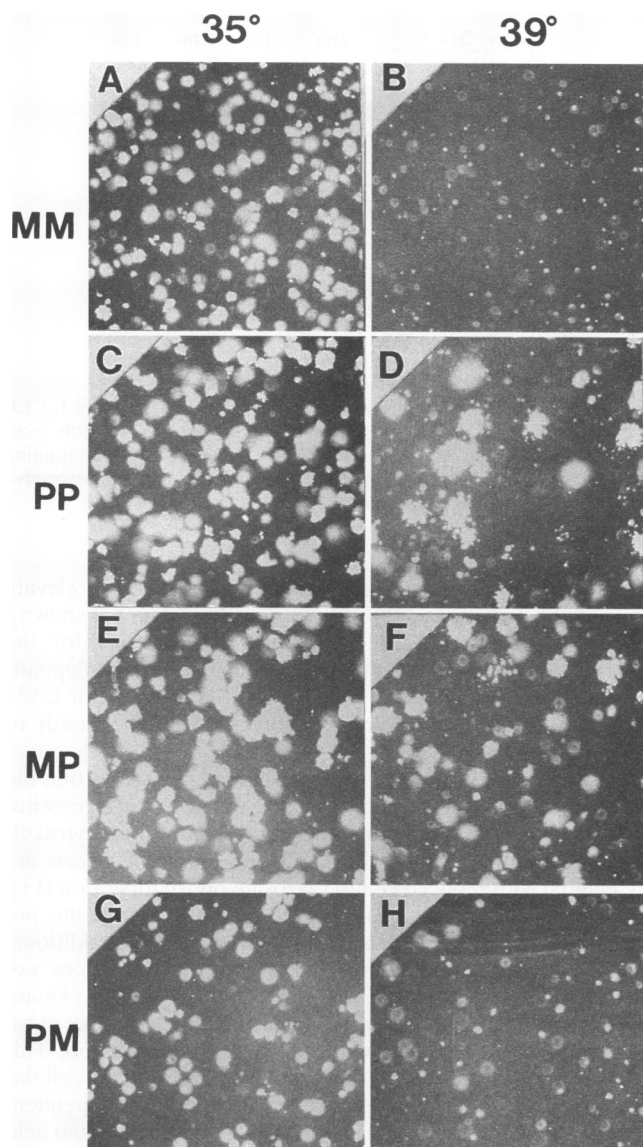


FIG. 3. Anchorage-independent growth of Rat-1 clones expressing chimeric v-Src molecules at permissive (35°C) and restrictive (39°C) temperatures. Colonies formed in soft agar (39) were photographed 10 to 14 days after seeding.

this assay (Fig. 4). There were no temperature-dependent variations in the relative distribution of any chimeric proteins, although the overall incorporation of label during the 3-h incubation tended to be higher at 41°C. Table 1 shows the mean cytoskeletal distribution of pp60^{src}, determined by densitometric scanning of three separate experiments. These figures overestimate the actual cytoskeletal levels of v-Src in all cases, since pp60^{c-src} formed a low background, which was mainly in the cytoskeletal compartment (Fig. 4). The cytoskeletal levels of MM protein were therefore lower than anticipated from the distribution determined by Dounce homogenization. It is thus possible that MM protein is concentrated at the cell periphery, as also suggested by immunofluorescence (29), but this association is more labile and the protein is more readily solubilized by MCSK buffer than is wild-type v-Src, a feature conferred by the G300V mutation.

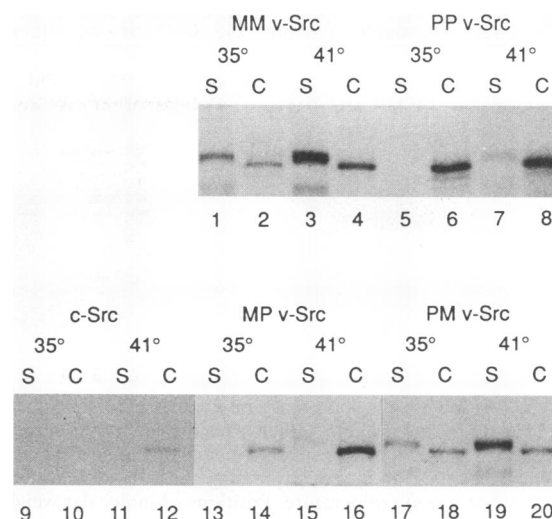


FIG. 4. Subcellular localization of v-Src. CEF labelled for 3 h with [³⁵S]methionine were separated into detergent-soluble (S; odd-numbered lanes) and cytoskeletal (C; even-numbered lanes) fractions, from which Src was immunoprecipitated. Lanes 9 to 12 are fractions from cells bearing the pRAV vector alone; the others express chimeras as indicated.

Biochemical effects of v-src mutations. In vitro kinase assays were performed with v-Src immunoprecipitated from CEF with monoclonal antibody 327, using acid-denatured enolase as the substrate. The results with similar quantities of proteins (Fig. 5A) showed considerable activity by PP and MP v-Src proteins and little activity above background on the part of MM and PM chimeras. Other sera, including polyclonal v-Src antisera from tumor-bearing rabbits, gave the same result, showing that it was not an artifact of the recognition of an SH3 domain epitope by monoclonal antibody 327 (2). Comparable results were obtained with v-Src proteins from transfected Rat-1 cells, and none of these proteins showed *ts* behavior in this assay (reference 2 and data not shown).

In spite of its constitutively reduced in vitro kinase activity, we have previously shown that MM v-Src protein can elevate intracellular phosphotyrosine levels in both morphologically transformed (35°C) and normal (41°C) CEF (28). In Rat-1 cells (Table 2), phosphotyrosine levels induced by L432 v-Src were also not *ts*, in contrast to the LA29 mutant protein, but they were two- to threefold lower than the relative levels induced by wild-type v-Src. This reflects exactly the behavior of these proteins in CEF, although Rat-1 cells had relative levels

TABLE 1. Cytoskeletal association of chimeric v-Src proteins

Chimera	% pp60 ^{src} in cytoskeletal fraction ^a	
	35°C	41°C
None (vector alone)	77 ^b	82 ^b
MM	33	25
PP	87	83
MP	83	90
PM	26	29

^a Determined by densitometric scanning and expressed as a percentage of total soluble plus cytoskeletal fractions. Values are means of three independent experiments of the type shown in Fig. 4.

^b pp60^{c-src} alone.

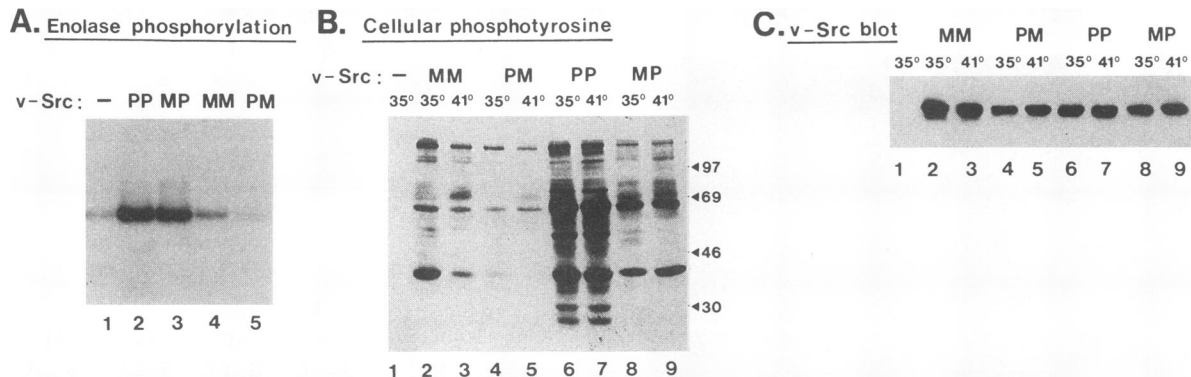


FIG. 5. (A) In vitro phosphotransferase activities of Src proteins from CEF at 35°C, using rabbit muscle enolase as the substrate. Lane 1, CEF containing pRAV vector alone. Other lanes display comparable amounts of chimeric v-Src as indicated. (B and C) Duplicate protein blots from CEF probed with either antiphosphotyrosine monoclonal antibody Py20 (B) or anti-v-Src monoclonal antibody EC10 (C). Lanes: 1, cells containing pRAV vector alone; 2 to 9, cells expressing chimeric v-Src as indicated and grown at either the permissive (even-numbered lanes) or restrictive (odd-numbered lanes) temperature. Positions of molecular weight markers (in kilodaltons) are shown to the right of panel B.

of phosphotyrosine that were universally about 10-fold lower than in counterpart CEF (28). These data present a paradox which may be explicable if the low MM kinase activity is an in vitro artifact or is nonetheless sufficient to increase the intracellular phosphotyrosine level. It is also possible that the elevation of the cellular phosphotyrosine level is due entirely or in part to the SH3 domain mutation, the v-Crk oncoprotein providing a precedent for a protein that increases tyrosine phosphorylation indirectly by way of its SH2 and SH3 domains (17).

To investigate these possibilities, we examined the ability of v-Src chimeras to elevate tyrosine phosphorylation in total cellular proteins (Fig. 5B). All of the chimeras increased phosphotyrosine levels, but to markedly different extents; since the levels of v-Src in these cells were comparable (Fig. 5C), the abilities to phosphorylate tyrosine were in the order PP > MP > MM > PM. This ranking accorded with the behavior of PP and MM proteins in Table 2 and may reflect in part the greater ability of PP and MP proteins to locate at the cytoskeleton (Fig. 4), but it also suggests that the low in vitro kinase activity associated with the G300V mutation (Fig. 5A) is not simply artifactual. The R107P lesion enhances tyrosine phosphorylation only in the context of a mutant residue 300 (chimera MM); to check whether this represents an independent activity of the v-Src SH3 domain, the MM kinase was fully inactivated by mutating the lysine at position 295 to arginine (references 11 and 26 and data not shown). This triple mutant was unable to induce morphological transformation or anchorage-indepen-

dent growth, and more significantly, it also failed to elevate tyrosine phosphorylation of cellular proteins (data not shown). Thus, although the R107P mutation compensates for the G300V lesion, any effect of the SH3 domain mutation depends on residual kinase domain activity. The SH3 domain of LA32 does not enhance tyrosine phosphorylation independently of the catalytic domain.

A *ts* target for LA32. The data presented above show that the G300V mutation in chimera PM is responsible for a constitutively low in vitro kinase activity (Fig. 5A) and poor cytoskeletal localization of v-Src (Fig. 4; Table 1). These effects are associated with reduced cellular tyrosine phosphorylation (Fig. 5B) which is sufficient to induce altered cell growth but not marked morphological changes (Fig. 2 and 3). The additional presence of the R107P mutation (chimera MM) does not significantly affect the subcellular localization or in vitro kinase activity of PM v-Src, but it enhances cellular tyrosine phosphorylation and restores morphological transformation. This transforming capacity is, however, *ts* (Fig. 1), whereas cellular tyrosine phosphorylation is not grossly temperature dependent (Table 2; Fig. 5B), in keeping with earlier phosphoamino acid analyses of LA32 v-Src (28). We have previously argued that LA32 is a minimal-deviation mutant, which differs only slightly in tyrosine phosphorylation at permissive and restrictive temperatures and thus has the potential to discriminate essential v-Src targets from adventitious substrates and those that may be necessary but are not sufficient for transformation (40). The present investigation substantiates that view and further suggests that the proposed subset of crucial targets, whose phosphorylation by LA32 v-Src is *ts*, includes those whose interactions with v-Src are enhanced by the SH3 domain mutation, thus conferring a transformed cell morphology in the presence of the G300V mutation. We therefore examined the effect of chimeric v-Src proteins on the function and tyrosine phosphorylation of a candidate target that binds to the v-Src SH3 domain, PI 3'-kinase (15).

The v-Src-associated activity of PI 3'-kinase was enhanced in chick cells expressing all of the chimeric v-Src proteins (Fig. 6A), but the enhancement by PM was relatively slight, and in common with the effect of PP, it was not temperature dependent. In contrast, expression of both MP and MM proteins was associated with a reduction of more than twofold in PI 3'-kinase activity at the restrictive temperature, although MP induced overall higher levels than MM. Thus, the activity of

TABLE 2. Relative abundance of phosphotyrosine in Rat-1 clones expressing v-Src

Clone	Relative abundance of phosphotyrosine ^a	
	35°C	39°C
Rat-1 fPrA (PP)	0.12	0.08
Rat-1 f29 (LA29)	0.036	0.019
Rat-1 f32 (MM)	0.037	0.048

^a Phosphoamino acids were scraped from cellulose plates, together with appropriate background areas. Phosphotyrosine levels are expressed as percentages of the total radioactivity in phosphoserine, phosphothreonine, and phosphotyrosine after subtracting background counts. Very similar results were obtained in three independent experiments. Levels in Rat-1 cells were not detectably above the background levels.

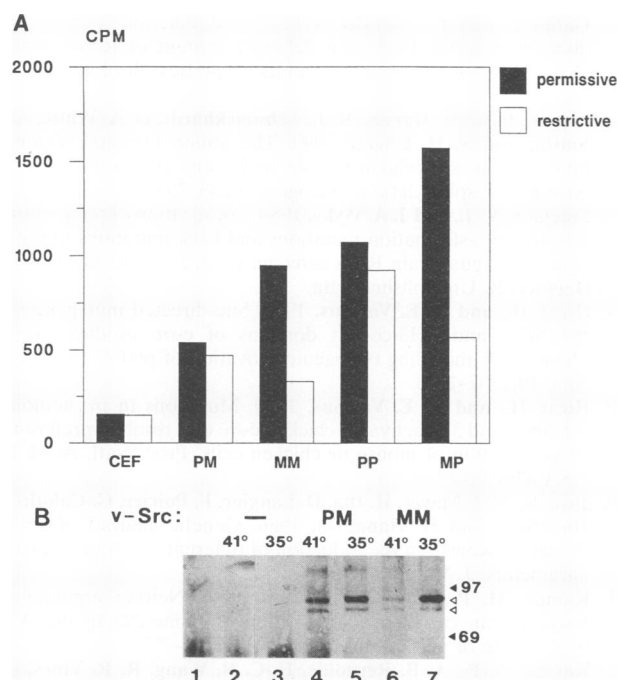


FIG. 6. (A) CEF PI 3'-kinase activity associated with indicated chimeric v-Src proteins at permissive and restrictive temperatures. Assays were performed as described in Materials and Methods, reaction products were separated by thin-layer chromatography, and the PI 3-phosphate band was scraped off the plate and counted. An example of one assay is shown, but comparable results were obtained in three independent determinations. (B) Blot of immunoprecipitated p85 subunit of PI 3'-kinase probed with antiphosphotyrosine monoclonal antibody Py20. Lanes: 1, CEF lysate immunoprecipitated with preimmune serum; 2 and 3, CEF containing pRAV vector alone; 4 to 7, CEF expressing the indicated v-Src chimeras. Lanes 3, 5, and 7 are samples from cells shifted to the permissive temperature from 41°C 16 h earlier. Positions of molecular weight markers (closed arrowheads) are shown (in kilodaltons) on the right, as are the positions of the two p85 species precipitated by the antiserum (open arrowheads).

this SH3-binding protein is enhanced, but in *ts* fashion, by the R107P mutation, whereas activation is constitutively diminished by the G300V lesion. The role of the R107P mutation was further demonstrated by the tyrosine phosphorylation of the p85 subunit of PI 3'-kinase (Fig. 6B). PM v-Src induced significant tyrosine phosphorylation of p85 at both restrictive and permissive temperatures, whereas tyrosine phosphorylation by the MM chimera was markedly *ts*.

DISCUSSION

Random mutagenesis of v-src, followed by selection of the desired phenotype, may give serendipitous information on the interplay of mutations that is not readily obtained by site-directed approaches. This is the case with LA32, in which the R107P and G300V mutations both contribute to the altered phenotype, although the effect of the SH3 lesion may have escaped detection in the absence of the kinase domain mutation.

The catalytic domain mutation. At both permissive and restrictive temperatures, the catalytic domain mutation drastically impairs in vitro tyrosine kinase activity and its consequences and reduces the ability of v-Src to associate with the cytoskeleton. DeClue and Martin (4) found that v-Src linker insertion mutations at amino acids 299 and 300 were, like

LA32, *ts* for transformation with a lowered, but not *ts*, in vitro kinase activity. They also found that mutant v-Src complex formation during biogenesis with the pp90 and pp50 proteins was higher at 41°C, but this latter phenomenon cannot explain the reduced MM cytoskeletal association, for LA32 v-Src complex formation is the same as for wild-type protein (29) and cytoskeletal association is not *ts* (Fig. 4). We have not, however, tested complex formation with the PM chimera, and it remains possible that the SH3 domain mutation in LA32 v-Src compensates for faulty complex formation mediated by the G300V mutation. With this caveat, we favor the probability that the G300V mutation increases the functional lability of v-Src, as revealed in both in vitro and in vivo investigations (Fig. 4 and 5). However, the mutant kinase domain retains some function, even at the restrictive temperature (Fig. 5B), and it can interact with the R107P mutation to elevate cell tyrosine phosphorylation, whereas the kinase inactive K295R mutation cannot.

The SH3 domain mutation. The SH3 domain contributes to the tight regulation of c-Src protein activity (20, 30), and mutations to c-Src SH3 tend to abrogate this regulation and enhance c-Src activity (6, 13, 23, 25). SH3 mutations in v-Src or activated c-Src proteins have shown either little effect or variously impaired cell transformation, agar growth, or kinase activity (8–10, 12, 35), although such studies are probably inherently biased toward the detection of deleterious effects. In the PA101 mutant of RSV, SH3 domain substitutions at residues 85 and 105 confer a fusiform morphology (18), and complex second-site mutations to residues 87 to 92, including a deletion of three amino acids, restore the round phenotype of the parental Bryan strain protein (5). To our knowledge, however, the LA32 R107P mutation is unique in enhancing the function of wild-type v-Src, for although its effect is striking in the context of the G300V mutation, which cripples catalytic domain function, it persists in MP chimeras, which are even more markedly transforming than the wild-type PP protein (Fig. 2).

Domain interactions. There is clearly an interplay between SH3 and catalytic domain mutations in LA32 v-Src which influences the integrated functions of the protein. The R107P mutation has no effect if the kinase is completely inactive, so it is not functioning as an adaptor module analogous to v-Crk. It may have an altered protein binding affinity or may bind a different spectrum of proteins which are thus exposed to, and enhance the effects of, the kinase domain activity, particularly in its enfeebled mutant form. Nuclear magnetic resonance studies have identified SH3 amino acids whose chemical shifts were changed by binding of candidate ligands (41), and substitutions of some of these putative ligand binding site residues alter SH3 function to variable extents (5, 6, 8, 9, 12, 23, 35). Residue 107 is, however, not implicated in ligand binding, in common with certain other residues whose substitutions nonetheless have marked biological consequences (8–10, 35). The E106K substitution and deletion of residue 105 studied by Wages et al. (35) both impair SH3 function in concert with a reduction in the v-Src-associated activity of PI 3'-kinase, a protein known to bind SH3 (15). Although this finding suggests that these mutations affect ligand binding, it does not prove it, and the R107P substituted SH3 domain clearly binds active PI 3'-kinase, albeit in a *ts* fashion (Fig. 6 and reference 7a). Moreover, modelling studies based on the SH3 structure of Yu et al. (41) indicate that the R107P change has no effect on the ligand binding site (19), but its proximity to the amino and carboxy extremities of SH3 may affect the linkage of SH3 to the rest of the v-Src protein. This prediction, which is testable by structural studies, raises the further

possibility that the function of the SH3 module is affected by its relationship with other v-Src domains. There could be an unchanged spectrum of proteins binding to the mutant SH3 region, but their disposition to the SH2 and catalytic domains could be altered, with consequent effects on their tyrosine phosphorylation (as in Fig. 6B) or the binding of v-Src to phosphotyrosine residues in the same or other proteins.

Temperature sensitivity of LA32. LA32 v-Src confers a *ts* transformed phenotype but elevates cellular phosphotyrosine at both permissive and restrictive temperatures, suggesting that LA32 temperature sensitivity is due to the conditional activation and/or tyrosine phosphorylation of a small number of crucial target molecules. These include PI 3'-kinase (Fig. 6) but not calpactin or vinculin, which are both phosphorylated to the same levels, independently of temperature (14, 28). Although we cannot conclude that the latter two molecules are irrelevant to transformation, since their phosphorylation may be a necessary but insufficient prerequisite, the data do imply an important role for PI 3'-kinase in transformation. The SH3 mutant studies of Wages et al. (35) correlate decreased association between PI 3'-kinase and v-Src with impaired morphological transformation, suggesting that PI 3'-kinase is important in regulating cell morphology. The poor activation of the enzyme at 41°C in fully transformed cells expressing MP v-Src (Fig. 6A) seems at odds with this suggestion, but the efficient membrane localization of MP v-Src (Fig. 4) may result in enough PI 3'-kinase activity at the appropriate cellular location to ensure transformation. We are testing this possibility.

Another question for further study is why LA32 v-Src effects on PI 3'-kinase are *ts* when there is no evidence for temperature sensitivity of either kinase activity in vitro or of tyrosine phosphorylation on other proteins (14, 28). It is possible that the *ts* effects of the LA32 mutations are apparent only when protein is bound to the SH3 or SH2 domain. Such v-Src molecules may themselves be a subset of proteins whose kinase activity depends largely on appropriate cellular contexts, since MM kinase functions well at both temperatures in vivo (Fig. 5B) but has poor activity in in vitro assays with total cell protein (Fig. 5A). These considerations further imply that changes to SH3 can alter the behavior of other v-Src domains, and we plan to exploit the temperature sensitivity of LA32 and its chimeras in investigating these interactions.

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